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Journal of Neuroimmunology 119 (2001) 1–9

Journal of  
Neuroimmunology

www.elsevier.com/locate/jneuroin

Sept

## Increased post-traumatic survival of neurons in IL-6-knockout mice on a background of EAE susceptibility

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Received 30 July 2000; received in revised form 28 March 2001; accepted 17 May 2001

### Abstract

Axonal injury initiates a process of neuronal degeneration, with resulting death of neuronal cell bodies. We show here that in C57BL/6J mice, previously shown to have a limited ability to manifest a post-traumatic protective immunity, the rate of neuronal survival is increased if IL-6 is deficient during the first 24 hours after optic nerve injury. Immunocytochemical staining performed 7 days after the injury revealed an increased number of activated microglia in the IL-6-deficient mice compared to the wild-type mice. In addition, IL-6-deficient mice showed an increased resistance to glutamate toxicity. These findings suggest that the presence of IL-6 during the early post-traumatic phase, at least in mice that are susceptible to autoimmune disease development, has a negative effect on neuronal survival. This further substantiates the contention that whether immune-derived factors are beneficial or harmful for nerve recovery after injury depends on the phenotype of the immune cells and the timing and nature of their dialog with the damaged neural tissue. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Neuroprotection; IL-6 deficiency; Axonal injury; Optic nerve; Glutamate toxicity; Inflammation

### 1. Introduction

Axonal injury initiates a process of neuronal degeneration that spreads both laterally and longitudinally (Faden, 1993; Faden and Salzman, 1992; McIntosh, 1993; Yoles and Schwartz, 1998). The fibers that are directly damaged by the injury degenerate and their cell bodies eventually die (Villegas-Perez et al., 1993). In addition, there is degeneration of fibers that escaped the primary lesion, caused by the activity of self-destructive mediators that emerge from the directly damaged fibers (Bazan et al., 1995; Liu et al., 1994; Lynch and Dawson, 1994). Degeneration resulting from the primary injury has been attributed to the deficiency of growth factors, normally supplied by the cellular targets of the fibers, as well as to the toxicity resulting from unfavorable buffering conditions caused by an imbalance of ions, excitatory amino acids, and other metabolic factors (Bazan et al., 1995; Liu et al., 1994; Lynch and Dawson, 1994).

Recent studies from our laboratory showed that both the lateral and the longitudinal spread of damage after axonal injury can be reduced by either passive transfer of autoimmune T cells (Hauben et al., 2000a,b; Moalem et al., 1999, 2000a,b) or active immunization with myelin-associated peptides (Fisher et al., 2001; Hauben et al., 2000b). This autoimmune neuroprotection is probably mediated by cytokines and trophic factors secreted by antigen-dependent activation of the T cells at the lesion site (Moalem et al., 2000a). With regard to the role of individual cytokines in the process of recovery from injury, the evidence is conflicting. Cytokines are viewed as key mediators in the pathogenesis of inflammatory lesions of the CNS, but analysis of their function is complicated by observations of both helpful and harmful effects (Bethea et al., 1999; Merrill and Benveniste, 1996).

IL-6, a multifunctional cytokine involved in the host response to infection, is produced by many different cell types including lymphocytes, monocytes, and parenchymal cells of various organs. In the CNS, astrocytes and microglia secrete IL-6 when infected with viruses or stimulated by cytokines (Frei et al., 1989; Lee et al., 1993). Both T cell-dependent and T cell-independent pathways of

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IL-6 production have been described in mice infected with different viruses (Frei et al., 1989). The intrathecal IL-6 response is thought to serve a dual function: (i) amplification of the immune-mediated clearance of virus, and (ii) neuronal repair. The latter assumption is based on the finding that nerve growth factor is produced by astrocytes stimulated by IL-6 (Frei et al., 1989). Furthermore, IL-6 was found to enhance survival of oligodendrocytes (Barres et al., 1993), although in order to be as effective as ciliary neurotrophic factor (CNTF) (Kahn and De Vellis, 1994) in this regard, it must be combined with its soluble receptor sIL-6R (Mendel et al., 1998). The latter is a natural agonist of IL-6 (Taga et al., 1989), and overexpression of IL-6 with sIL-6R was shown to increase nerve regeneration in transgenic mice (Hirota et al., 1996). However, IL-6 may also cause neurotoxicity, as evidenced by the neuronal degeneration that develops in transgenic mice that overexpress IL-6 in the CNS parenchyma (Campbell et al., 1993).

Recent evidence indicates that IL-6 modulates experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease induced by immunization with myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). Whereas the encephalitogenic peptide pMOG 35–55 induces EAE in H-2<sup>b</sup> mice in which the IL-6 gene is intact, IL-6-deficient mice are resistant to EAE (Mendel et al., 1998).

Studies in our laboratory have demonstrated that spontaneous recovery from optic nerve injury is critically influenced by the animals genetic background. The survival rate of RGCs after optic nerve injury in EAE-susceptible mouse strains such as C57BL/6J mice was significantly lower than in EAE-resistant strains (Kipnis et al., 2001). In the present study we show that the survival rate in an EAE-susceptible mouse strain is increased in the absence of IL-6. Furthermore, the critical post-injury period during which IL-6 deficiency can improve the survival rate is the first 24 h, as the outcome is not affected by the presence or absence of IL-6 after that time. We further show that the higher survival rate following axonal injury in the IL-6-deficient mice does not correlate with a reduced inflammatory response. On the contrary, 7 days after optic nerve injury, a higher level of Mac-1 immunoreactivity (an indicator of microglial activation) was observed in IL-6-deficient mice.

## 2. Materials and methods

### 2.1. Animals

Male and female C57BL/6 × 129Sv (H-2<sup>b</sup>) mice (controls) and homozygous IL-6<sup>-/-</sup> mice (8–12 weeks old) were obtained from the Weizmann Institute of Science, Israel. The mice were housed in a light- and temperature-controlled room and matched for age in each experiment.

### 2.2. Antigens

Rat-derived MOG peptides 1–22 (GQFRVIGPGHPIRALVGDEAEL) were synthesized in the laboratory of Prof. M. Fridkin at the Department of Chemistry of the Weizmann Institute of Science, using the Fmoc technique with an automatic multiple peptide synthesizer (AMS422, Abimed, Langenfeld, Germany). Ovalbumin (OVA), fraction V, was purchased from Sigma (Rehovot, Israel).

### 2.3. Active immunization

Mice were immunized subcutaneously at one site in the flank with 200 µl of emulsion consisting of MOG 1–22 or OVA (300 µg per mouse), emulsified in complete Freund's adjuvant (CFA) supplemented with 500 µg of *Mycobacterium tuberculosis*.

### 2.4. T cell lines

A T cell line was generated from draining lymph node cells obtained from H-2<sup>b</sup> mice immunized with the MOG 1–22 antigen described above. Draining lymph nodes from immunized mice were removed 11 days after immunization and pooled in ice-cold PBS. A single-cell suspension was prepared and fractionated on plastic tissue-culture dishes, and the non-adherent cells ( $3 \times 10^6$ /ml) and irradiated (2000 rad) syngeneic normal spleen cells ( $5 \times 10^6$ /ml) were suspended in proliferation medium (Ben-Nun and Lando, 1983) in 60-mm petri dishes (6 ml per dish). For selection of lymphoblasts responding to MOG, the cultured cells were supplemented with MOG (25 µg/ml). After incubation for 72 h, the cultures were collected and washed, and the cells ( $4 \times 10^5$ /ml) were resuspended in propagation medium (Ben-Nun and Lando, 1983) and reseeded in petri dishes. The cultures were maintained in propagation medium with changes of medium or splitting of culture for 8–10 days, and the lymphoblasts ( $2 \times 10^5$ /ml) were then restimulated with MOG (25 µg/ml) in the presence of irradiated syngeneic normal spleen cells ( $10 \times 10^6$ /ml). The cell line was maintained in cycles of alternate stimulation with the MOG peptide and propagation.

### 2.5. Glutamate toxicity to mouse RGCs

The right eyes of anesthetized mice were punctured with a 27-gauge needle in the upper part of the sclera, and a 10-µl Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Mice were injected with a total volume of 1 µl (200 nmol) of L-glutamate dissolved in saline.

### 2.6. Labeling of RGCs and assessment of their survival

For baseline labeling of RGCs, a stereotactic dye was applied prior to the crush injury. Eleven days after the first

active immunization, mice were deeply anesthetized by intraperitoneal injection of xylazine (14 mg/kg; Vitamed, Bat Yam, Israel) and ketamine (60 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and placed in a small stereotactic instrument. The skull was exposed and kept dry and clean using 3% hydrogen peroxide. The bregma was identified and marked. A hole was drilled above the superior colliculus of each hemisphere (0.292 mm behind and 0.05 mm lateral to the midline). Using a stereotactic measuring device and a Hamilton injector, the mice were injected with FluoroGold (3% in saline, Fluorochrome, Denver, CO; 1  $\mu$ l) at one site in the superior colliculus of each hemisphere, at a depth of 0.16 mm from the bony surface of the brain. After completion of the injection, the wound was sutured. Retrograde uptake of the dye provides a marker of the living cells. The same procedure of labeling was applied in mice exposed to glutamate toxicity, 4 days after glutamate injections.

Three days after dye application, the right optic nerve of each mouse was subjected to a crush injury severe enough to cause primary damage to all the axons. After such an injury, the number of labeled cell bodies at a given time provides an indication of the rate of secondary degeneration and cell body death.

Two weeks after the crush injury and one week after glutamate toxicity, the mice were killed. Eyes showing signs of ischemia or slight infection were discarded, and only eyes that looked healthy were used. Each retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy. Labeled RGCs from five to six fields of identical size, located at approximately the same distance from the optic disk, were counted under the fluorescence microscope and averaged. Calculation of the number of labeled RGCs per square millimeter in the retina of the injured eye provided a quantitative measure of the total degeneration.

### 2.7. Crush injury of mouse optic nerve

Three days after stereotactic dye application (14 days after the first active immunization), the mice were deeply anesthetized by intraperitoneal injection of xylazine (14 mg/kg) and ketamine (60 mg/kg). Using a binocular-operating microscope, the conjunctiva of the right eye was incised and the optic nerve was exposed. With the aid of cross-action forceps, the optic nerve was subjected to a severe crush injury 1–2 mm from the eyeball. The uninjured contralateral nerve was left undisturbed.

### 2.8. Immunocytochemistry

Longitudinal cryosections of the excised nerves (10  $\mu$ m thick) were placed on gelatin-coated glass slides and frozen until preparation for fluorescence staining. Sections were fixed in ethanol for 10 min at room temperature and then

in acetone for 10 min (for MOMA-2 staining), washed twice in double-distilled water, and incubated for 3 min in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20). Sections were then incubated for 1 h at room temperature with rat antibody to mouse macrophages (MOMA-2, Serotec, Oxford, England), mouse monoclonal antibody to mouse neurofilaments (N52, Sigma) diluted 1:200, rat antibody to activated microglia (Mac-1, Pharmingen) diluted 1:50 or rat antibody to B7.2 (CD86, Pharmingen) diluted 1:20 in PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were washed three times with PBS containing 0.05% Tween-20 and then incubated with Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), goat anti-mouse IgG (Jackson ImmunoResearch) or FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch) for 1 h at room temperature. They were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane to inhibit quenching of the fluorescence. Sections were viewed with a confocal microscope. Staining in the absence of the first antibodies was used as a negative control.

### 2.9. Statistical analysis

The number of RGCs per square millimeter was calculated for each experiment. Statistical analysis was performed by one-way ANOVA.

## 3. Results

### 3.1. Increased resistance to secondary degeneration in IL-6-deficient mice

We recently showed that vaccination with the non-encephalitogenic myelin-associated peptide MOG 1–22 reduces degeneration of retinal ganglion cells (RGCs) after optic nerve injury in C3H.SW (H-2<sup>b</sup>) mice (Fisher et al., 2001), which are susceptible to EAE (Mendel et al., 1995). C57BL/6J mice, which are also susceptible to EAE, are known to have a limited ability to manifest a T-cell mediated protective autoimmunity after CNS injury. In the present study, we first examined whether MOG vaccination would be beneficial in C57BL/6J mice, which because of a genetically engineered deficiency in IL-6, are resistant to EAE. Two weeks prior to the crush injury, wild-type and IL-6 knockout (IL-6<sup>-/-</sup>) mice were immunized with MOG 1–22 or with OVA (a non-self antigen) as a control. Three days before the injury, their RGCs were retrogradely labeled by stereotactic application of FluoroGold. Two weeks after the injury, the retinas were excised and the labeled RGCs were counted. As shown in Fig. 1a, the number of surviving RGCs in the wild-type mice actively immunized with MOG 1–22 ( $1101 \pm 101$ ,  $n = 4$ ) was significantly greater than in those immunized with

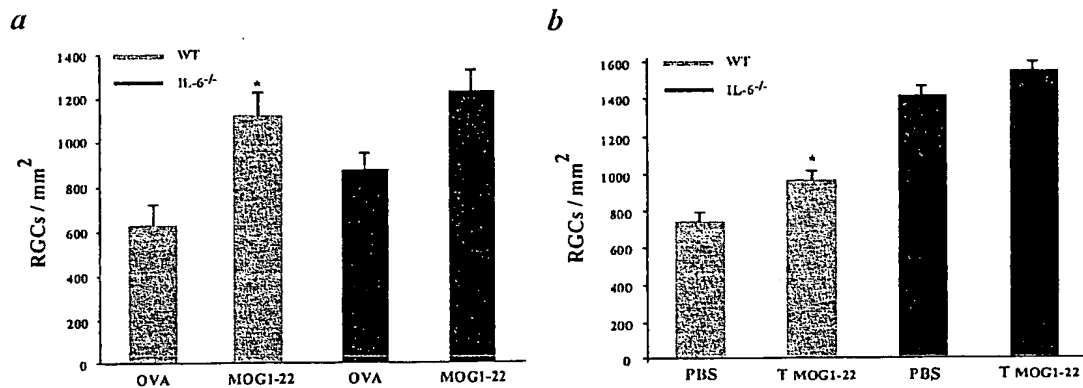


Fig. 1. Post-traumatic survival of RGCs is significantly higher in IL-6-deficient mice than in wild-type mice. The histograms represent the mean numbers of labeled RGCs per square millimeter  $\pm$  SEM. (a) IL-6-deficient (IL-6<sup>-/-</sup>) and control wild-type (WT) mice were injected with pMOG 1–22 or OVA, 14 days prior to optic nerve injury. The neurotracer dye, FluoroGold, was applied stereotactically 3 days prior to injury. Two weeks after the injury, the retinas were excised and flat-mounted. Labeled RGCs from five randomly selected fields in each retina (all located 1 mm from the optic disk) were counted by fluorescence microscopy. The mean number of labeled RGCs in the retinas of WT mice pretreated with pMOG 1–22 ( $1101 \pm 101$ ,  $n = 4$ ) was significantly higher ( $p < 0.01$ , one-way ANOVA) than in mice pretreated with OVA ( $610 \pm 94$ ,  $n = 4$ ). The mean number of labeled RGCs in the retinas of IL-6<sup>-/-</sup> mice pretreated with pMOG 1–22 ( $1204 \pm 113$ ,  $n = 6$ ) did not differ significantly ( $p > 0.05$ , one-way ANOVA) from that in mice pretreated with OVA ( $857 \pm 89$ ,  $n = 8$ ). (b) IL-6<sup>-/-</sup> and WT mice were injected with T cells specific to MOG 1–22 or PBS immediately after the injury. Dye application, preparation, counting of RGCs, and calculation of RGC survival were as described for (a). The mean number of labeled RGCs in the retinas of WT mice injected with T<sub>MOG 1–22</sub> ( $941 \pm 54$ ,  $n = 18$ ) was significantly higher ( $p < 0.01$ , one-way ANOVA) than in mice injected with PBS ( $726 \pm 36$ ,  $n = 16$ ). The mean number of labeled RGCs in the retinas of IL-6<sup>-/-</sup> mice injected with T<sub>MOG 1–22</sub> ( $1518 \pm 40$ ,  $n = 18$ ) did not differ significantly ( $p > 0.05$ , one-way ANOVA) from that in mice injected with PBS ( $1387 \pm 47$ ,  $n = 12$ ). Note the extremely significant difference in RGC survival rates between the PBS-injected IL-6<sup>-/-</sup> mice and the PBS-injected WT mice ( $p < 0.001$ , one-way ANOVA).

OVA ( $610 \pm 94$ ,  $n = 4$ ;  $p < 0.05$ , one-way ANOVA). In contrast, the difference between MOG-immunized and OVA-immunized IL-6<sup>-/-</sup> mice ( $1204 \pm 113$ ,  $n = 6$  and  $857 \pm 89$ ,  $n = 8$ , respectively) was not significant ( $p > 0.05$ , one-way ANOVA). The number of viable RGCs 2 weeks after the crush injury in wild-type and IL-6<sup>-/-</sup> mice injected immediately after the injury with T cells specific to MOG 1–22 (T<sub>MOG 1–22</sub>) is shown in Fig. 1b. Significantly more RGCs were viable in the wild-type mice injected with T<sub>MOG 1–22</sub> ( $941 \pm 54$ ,  $n = 18$ ) than in those injected with PBS ( $726 \pm 36$ ,  $n = 16$ ;  $p < 0.01$ , one-way ANOVA). The corresponding difference in the IL-6<sup>-/-</sup> mice ( $1518 \pm 40$ ,  $n = 18$  and  $1387 \pm 47$ ,  $n = 12$ , respectively) was not significant ( $p > 0.05$ , one-way ANOVA). Thus, as expected (Fisher et al., 2001), both active and passive immunization with a MOG epitope led to a significantly increased survival of RGCs in the wild-type mice. Interestingly, the RGC survival rate was significantly higher in the IL-6<sup>-/-</sup> mice injected with PBS ( $n = 12$ ) than in the PBS-injected wild-type mice ( $n = 16$ ;  $p < 0.001$  one-way ANOVA), but in contrast to the wild-type, MOG immunization, active as well as passive, had no effect in the IL-6<sup>-/-</sup> mice.

### 3.2. Reversal of neuroprotection in IL-6-deficient mice by a single IL-6 injection

To confirm that the higher survival rate in the IL-6-deficient mice is directly related to the absence of IL-6, we attempted to reverse the presumed effect of IL-6 defi-

ciency by injecting both IL-6<sup>-/-</sup> and wild-type mice intraperitoneally with IL-6 immediately after the injury (Fig. 2). Injection of IL-6 (compared to PBS injection as a

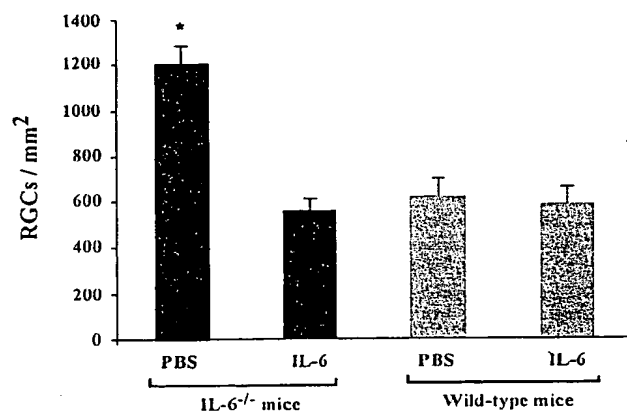


Fig. 2. Systemic injection of IL-6 immediately after injury reverses the neuroprotective effect of IL-6 deficiency. The histograms record the mean numbers of labeled RGCs per square millimeter  $\pm$  SEM. IL-6<sup>-/-</sup> and WT mice were injected with IL-6 or PBS immediately after the injury. Dye application, preparation, counting of RGCs, and calculation of RGC survival were as described for Fig. 1. The mean number of labeled RGCs in the retinas of WT mice injected with IL-6 ( $576 \pm 68$ ,  $n = 6$ ) did not differ ( $p > 0.05$ , one-way ANOVA) from that in mice injected with PBS ( $606 \pm 78$ ,  $n = 10$ ). In contrast, the mean number of labeled RGCs in the retinas of IL-6<sup>-/-</sup> mice injected with IL-6 ( $547 \pm 67$ ,  $n = 4$ ) was significantly lower ( $p < 0.001$ , one-way ANOVA) than that in mice injected with PBS ( $1201 \pm 88$ ,  $n = 12$ ).

control,  $n = 10$ ) reduced the RGC survival rate in IL-6<sup>-/-</sup> mice ( $n = 12$ ) from 1201 to 547 per mm<sup>2</sup> ( $p < 0.001$ , one-way ANOVA). No significant effect of IL-6 injection was observed in the wild-type mice.

The effect of the injected IL-6 in the IL-6<sup>-/-</sup> mice raised questions about the time period after injury when the absence or presence of IL-6 is critical for neuronal survival. In other words, it was important to find out at what stage the absence of IL-6 is critical for survival. To address this question, IL-6<sup>-/-</sup> mice were injected with IL-6, 1 or 3 days after the injury, and the number of surviving RGCs 2 weeks after the injury was compared with that in mice injected with IL-6 immediately after the injury (Fig. 3). The number of surviving RGCs in IL-6-deficient mice injected with IL-6 1 day after the injury was 1072 per mm<sup>2</sup> ( $n = 7$ ), compared to 547 per mm<sup>2</sup> in the mice injected immediately after the injury, indicating that injection of IL-6 as early as 1 day after the injury was already too late to reverse the endogenous protective mechanism resulting from the IL-6 deficiency. A similar tendency was seen in the mice injected 3 days after injury ( $n = 5$ ).

### 3.3. Increased resistance to glutamate toxicity in IL-6-deficient mice

To determine whether the higher survival rate of RGCs after injury in the IL-6-deficient mice is restricted to mechanical injuries or is common to any CNS insult, we compared the outcome of a direct biochemical insult caused by glutamate toxicity in IL-6-deficient and wild-type mice.

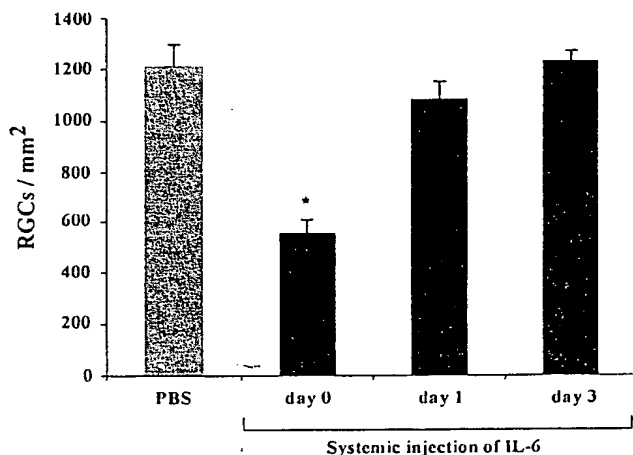


Fig. 3. Delayed injection of IL-6 does not reverse the neuroprotective effect of IL-6 deficiency. The histograms represent the mean numbers of labeled RGCs per square millimeter  $\pm$  SEM. IL-6<sup>-/-</sup> mice were injected with IL-6 immediately after the injury ( $n = 10$ ) and again 1 day ( $n = 7$ ) and 3 days later ( $n = 5$ ). Dye application, preparation and counting of RGCs, and calculation of RGC survival were as described for Fig. 1. Note, there is almost no difference in RGC survival between mice injected with IL-6 3 days after the injury ( $1214 \pm 56$ ) and mice injected with PBS immediately after the injury ( $1201 \pm 88$ ).

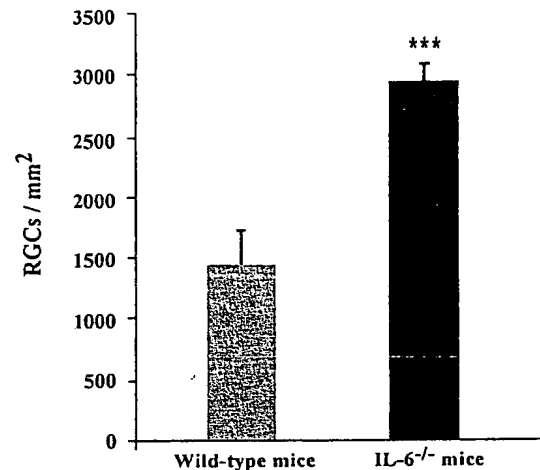


Fig. 4. RGC survival from glutamate toxicity is significantly higher in IL-6-deficient mice than in wild-type mice. The histograms represent the mean numbers of labeled RGCs per square millimeter  $\pm$  SEM. IL-6-deficient (IL-6<sup>-/-</sup>) and control wild-type (WT) mice were exposed to glutamate toxicity, as described in Section 2. The neurotracer dye Fluoro-Gold was applied stereotactically 3 days after the glutamate injection. One week after the glutamate insult, the retinas were excised and flat-mounted. Labeled RGCs from five randomly selected fields in each retina (all located 1 mm from the optic disk) were counted by fluorescence microscopy. The mean number of labeled RGCs in the retinas of IL-6<sup>-/-</sup> mice ( $2920 \pm 106$ ,  $n = 7$ ) was significantly higher ( $p < 10^{-7}$ , Student's *t*-test) than in wild-type ( $1419 \pm 64$ ,  $n = 6$ ).

L-glutamate (200 nmol) was intravitreally injected into the right eyes of IL-6-deficient and wild-type mice. Three days after the insult, all RGCs were stereotactically labeled. One week later, the retinas were excised and the labeled RGCs were counted. As shown in Fig. 4, the number of surviving RGCs was significantly higher in IL-6-deficient mice ( $n = 7$ ) than in the wild-type mice ( $n = 6$ ;  $p < 10^{-7}$ , Student's *t*-test; Fig. 4). These results suggest that in the absence of IL-6, neurons survive better after an insult, irrespective of its nature.

### 3.4. Macrophage accumulation at the injury site in wild-type and IL-6<sup>-/-</sup> mice

Conflicting effects on traumatized neuronal tissue have been reported with respect to both macrophages and T cells. It was therefore important to determine whether the relatively favorable outcome of optic nerve injury in mice deficient in IL-6 is accompanied by any change in the amount of invading T cells and/or macrophages. One week after optic nerve injury, the time of peak accumulation of inflammatory cells, we excised nerves from both wild-type and IL-6<sup>-/-</sup> mice. In both types of mice, staining with anti-neurofilament (NF) antibodies to delineate the injury site and with MOMA-2 antibodies to visualize macrophages showed that macrophages had accumulated at the site of injury (Fig. 5a,b,d,e). More macrophages had

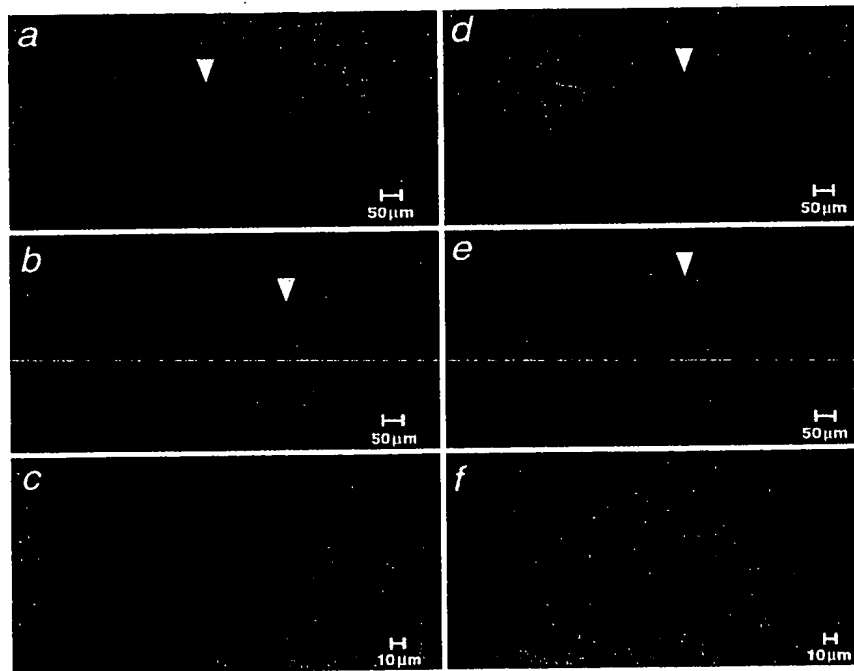


Fig. 5. Macrophage accumulation at the injury site in wild-type and IL-6<sup>-/-</sup> mice. Seven days after the injury, the optic nerves of wild-type (a, b, c) and IL-6<sup>-/-</sup> (d, e, f) mice were excised and labeled immunocytochemically. Serial optic nerve sections immunolabeled for neurofilaments (a, d) delineate the site of injury (designated by the arrows). Immunolabeling for MOMA-2 (b, c, e, f) indicates the presence of macrophages at the injury site.

accumulated in the IL-6<sup>-/-</sup> mice than in wild-type (Fig. 5b,e). No difference was observed in the numbers of accumulated T cells (data not shown) in the injured optic nerves of the two groups of mice.

The observation that more macrophages accumulated in the injured optic nerve of mice deficient in IL-6 prompted us to examine whether the difference in the activation state

of macrophages and microglia in IL-6-deficient and wild-type mice. This was done by analyzing macrophage/microglial expression of Mac-1 (C3 complement receptor) and B7.2, as indicators of microglial activation (Jensen et al., 2000; Koshinaga et al., 2000; Fiske and Brunjes, 2000). One week after the injury, Mac-1 immunoreactivity was detected at the injury site of both IL-6-deficient and

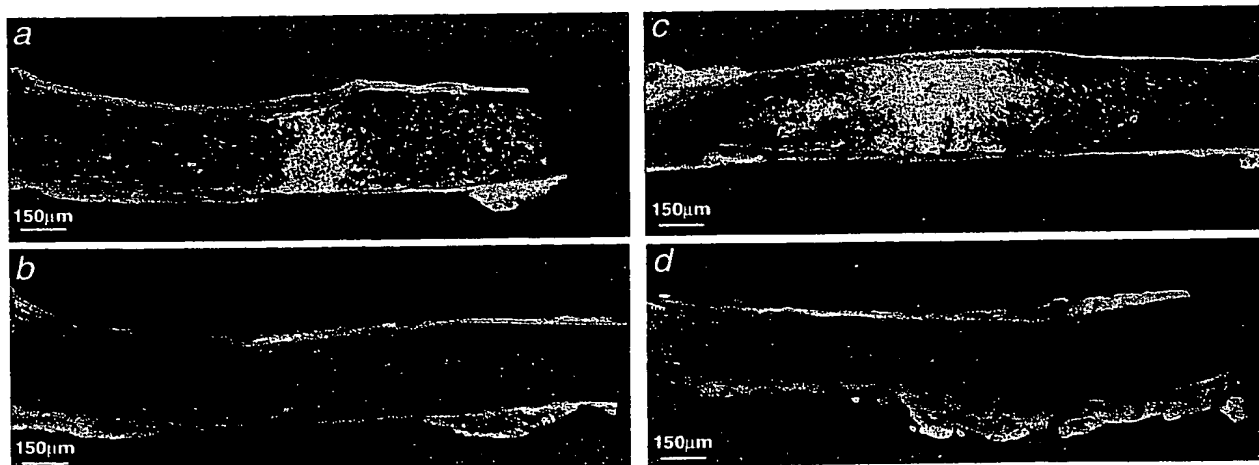


Fig. 6. Intense Mac-1 expression at the injured optic nerves of IL-6-deficient mice. Seven days after the injury, the optic nerves of wild-type (a, b) and IL-6<sup>-/-</sup> (c, d) mice were excised and labeled immunocytochemically. Optic nerve sections immunolabeled for Mac-1 (a, c) indicate the presence of activated microglial cells. Immunolabeling for B7.2 (b, d) indicates the expression of B7.2 molecules at the injured optic nerve.

wild-type mice, but was much more pronounced in the IL-6-deficient mice (Fig. 6a,c). No differences in B7.2 immunoreactivity were observed between injured optic nerves of the IL-6-deficient and wild-type mice (Fig. 6b,d).

#### 4. Discussion

The results of this study suggest that mice deficient in IL-6 are better endowed with a physiological ability to cope with stressful conditions of CNS injury than wild-type mice.

The physiological functions of IL-6 in the CNS are known to be complex. Numerous studies have indicated that IL-6 exerts multiple effects, both beneficial and destructive, on CNS cells (Van Wagoner and Benveniste, 1999). In this study, we demonstrated an increased neuronal survival rate in IL-6-deficient mice, assessed 2 weeks after optic nerve injury or 1 week after intravitreal injection of toxic amounts of glutamate. Interestingly, in a recent study, IL-6-deficient mice showed a decrease in the number of activated brain macrophages associated with brain focal cryo-injury (Penkowa et al., 1999), suggesting a role for IL-6 in the control CNS inflammation. Moreover, dysregulation and overexpression of IL-6 are known to contribute to the neuropathology and pathophysiology associated with many diseases (Gadient and Otten, 1997). Experiments using various transgenic mouse models point to the destructive potential of dysregulated IL-6 in the CNS. Use of one of these models led to the suggestion that overproduction of IL-6 in the CNS may ultimately result in increased central production of inflammatory cytokines, thus supporting a proinflammatory and detrimental role of IL-6 when dysregulated in the CNS (Di Santo et al., 1996).

The absence of IL-6 during the first 24 h after the injury was shown here to be beneficial for neuroprotection. One possible explanation is that IL-6 deficiency within the appropriate time window might affect the nature of activation acquired by the microglia, thereby possibly allowing the creation of protective conditions at a critical stage for post-traumatic neuronal survival. This interpretation may be consistent with reports of impaired neuronal regeneration observed in leukemia inhibitory factor (LIF) knock-out mice (Sugiura et al., 2000). It might also be in line with a recent work showing that IL-10 promotes functional recovery after spinal cord injury (Bethua et al., 1999), provided that it is supplied immediately after injury; when administered late, it has adverse effects. The apparently higher level of microglial/macrophage activation seen after injury in the IL-6-deficient mice than in the wild-type mice, as observed in the present study, argues in favor of changes in the phenotype of the inflammatory cells or the kinetics of the inflammatory reaction rather than in the amount of inflammation. It is also possible that the reaction is influenced by the existence of a mechanism, unrelated to the immune activity of the IL-6-deficient mice, which attenu-

ates post-traumatic auto-destructive processes or increases the organism's post-traumatic resistance to them.

The fact that the IL-6-deficient mice are although endowed, with a better endogenous protection against stressful conditions than the wild type mice do not develop EAE, may suggest that they mount beneficial autoimmunity rather than destructive autoimmunity as a result of the injury. Such interpretation is in line with our contention of the inverse relationship between susceptibility to develop an autoimmune disease and ability to mount beneficial autoimmunity (Yoles et al., 2001; Kipnis et al., 2001).

The results of the present study, strongly suggest that if neuroprotective treatment is undertaken using a single therapeutic factor (monotherapy), its timing must be carefully monitored, otherwise it can be more destructive than helpful. Moreover, ongoing treatment with one factor would appear to be risky, as the post-traumatic needs for recovery change with time. A more promising approach may be a comprehensive cell therapy, like the one potentially provided by autoimmune T cells (Moalem et al., 1999, 2000a,b; Hauben et al., 2000a,b), with continuous release of various factors at the site of injury. It seems reasonable to assume that the timing and dynamics of release of such factors would be in accordance with the needs of the tissue (Schwartz and Cohen, 2000; Schwartz et al., 1999).

Recently, we showed that active immunization with non-encephalitogenic myelin-associated peptides, such as MOG 1–22, increases the RGC survival rate 2 weeks after optic nerve crush injury in C3H.SW (H-2<sup>b</sup>) mice (Fisher et al., 2001). The endogenous mechanism of neuroprotection awakened by injury in our IL-6-deficient mice, which also possess the H-2<sup>b</sup> haplotype, could be only slightly boosted by passive or active immunization with MOG 1–22 peptide, whereas the same types of immunization were significantly effective in the wild-type mice. The insignificant effect of the MOG immunization in the IL-6-deficient mice might be attributable to their higher basal level of RGC survival compared to that of the wild-type mice. It is possible that in the absence of IL-6, whose activity normally evokes a Th-1 type response (Mosmann and Coffman, 1989), the spontaneous response to the injury is differently regulated. Such regulation appears to be critical for beneficial autoimmunity to be manifested (Schwartz and Kipnis, 2001). Alternatively, in the absence of IL-6 the damaged neural tissue might differently express cytokines, chemokines or other immune-related compounds and their receptors that potentially contribute to the local immune response. One such molecule is B7.2, which was recently found by our group to be a better candidate for a type of cross talk with T cells that produces a beneficial effect (Butovsky et al., 2001). No differences were found here in B7.2 expression, 1 week after injury, between wild-type and IL-6-deficient mice. This should be further examined at earlier time points after the injury. The increased rate of RGC survival, which was observed here in IL-6 deficient

mice with a background of EAE susceptibility, might be unique to susceptible strains rather than generally applicable. It may be related to the fact that autoimmunity needs to be kept under tight control, a requirement accomplished here by the effect of the IL-6 deficiency. The increased RGC survival rate in these mice was indeed found to be correlated with the increased resistance to EAE. Further studies should be carried out to determine whether IL-6 deficiency in genetically resistant EAE mice will lead to a similar outcome in terms of post-traumatic RGC survival.

It remains to be determined whether the observed effect of the IL-6 is related to T cells and in particular whether destructive and beneficial autoimmunity are mediated by identical, though differently regulated T cells or by distinctive T cells. In either case, the results of the present study could be interpreted as showing that transient deficiency in IL-6 is beneficial for survival of the cell bodies of traumatized CNS axons. In addition, the increased neuronal resistance of the IL-6-deficient mice to both mechanical and biochemical insults, as found in the current study, might represent a more general phenomenon of resistance to a wide range of injurious conditions. We believe that the results of this study may have profound implications for neurodegenerative disorders in general, and those associated with optic nerve degeneration (Schwartz et al., 1996, 1999; Schwartz and Kipnis, 2001).

## Acknowledgements

We thank S. Smith for editorial assistance and A. Shapira for animal assistance. M. Schwartz holds the Maurice and Ilse Katz Professorial Chair in Neuroimmunology. The work was supported in part by the Glaucoma Research Foundation awarded to M.S.

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